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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68, C07K 14/32 — A1

(11) International Publication Number: WO 98/03678

(43) International Publication Date: 29 January 1998 (29.01.98)

(21) International Application Number: PCT/GB97/01987

(22) International Filing Date: 24 July 1997 (24.07.97)

(30) Priority Data:
9615516.3 24 July 1996 (24.07.96) GB

(71) Applicant *(for all designated States except US)*: BIOTEC LABORATORIES LIMITED [GB/GB]; 32 Anson Road, Martlesham Heath, Ipswich, Suffolk IP5 7RG (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: STEWART, Gordon, Sydney, Anderson, Birnie [GB/GB]; 14 James Avenue, Loughborough, Leicestershire LE11 0QL (GB). FRANCIS, Kevin, Patrick [GB/GB]; 40 Elizabeth Avenue, North Hykeham, Lincoln, Lincolnshire LN6 9RR (GB). SCHERER, Siegfried [DE/DE]; Fruehlingstrasse 67a, D-85354 Freising (DE). LECHNER, Sabine [DE/DE]; Am Hoechberg 59, D-97234 Reichenberg (DE). MAYR, Ralf [DE/DE]; Angermeierstrasse 53, D-85356 Freising (DE).

(74) Agent: PRIVETT, Kathryn, L.; Stevens, Hewlett & Perkins, 1 Serjeants' Inn, Fleet Street, London EC4Y 1LL (GB).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: DETECTION OF PSYCHROTROPHIC *BACILLUS*

(57) Abstract

A method for detecting psychrotrophic *Bacillus* in a sample is described, which method comprises determining whether the sample contains a nucleic acid which codes for a *Bacillus* major cold shock protein or a major cold protein homologue and which has a conserved nucleotide base which is different in psychrotrophic and mesophilic strains of *Bacillus*. The method is of particular use for detecting the presence of food spoilage organisms in food samples.

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Detection of Psychrotrophic *Bacillus*

This invention concerns differentiating between strains of *Bacillus* (e.g. *B. cereus*), in particular between psychrotrophic and mesophilic strains.

Bacillus cereus and other *Bacillus* species such as *Bacillus mycoides* (previously known as *Bacillus cereus* var. *mycoides*) are bacteria associated with the spoilage of food products stored at refrigeration temperature. Spoilage of milk and other dairy products by this organism is a particular problem. The presence of *Bacillus cereus* in food products can be tested for by a well known biochemical test (API test) and affected food batches destroyed. However, this can be wasteful since certain strains of *Bacillus*, described as mesophilic, do not grow at refrigeration temperatures and therefore are not generally involved in spoilage of food stored at such temperatures. It is the psychrotrophic *Bacillus* strains, which do grow and proliferate at low temperatures such as the standard refrigeration temperature of 4 - 7°C, that will cause food to spoil. According to standard terminology, bacteria are psychrotrophic if they will grow and proliferate when left for ten days at 7°C or below.

Psychrotrophic bacteria have been recognised as a recurring problem in the refrigerated storage and distribution of fluid milk and other perishable dairy products. Although much emphasis has been focused on post-pasteurisation contaminants such as *Pseudomonas*, improved processing conditions for milk and other dairy products has meant less interest in these non-heat-resistant contaminants and more attention being directed at psychrotrophic sporeformers such as *Bacillus*. It has been estimated that 25% of all shelf-life problems associated with conventionally pasteurised milk and cream products in the USA may be linked to thermoduric psychrotrophs, with the majority of these contaminants being psychrotrophic *Bacillus* (e.g. *B. cereus*). Moreover, the incidence of

psychrotrophic *Bacillus* species that are associated with the spoilage of dairy products is even greater in a number of European countries where the average storage temperature of many of these milk products is several degrees higher.

Therefore, it would be a significant advantage to have a test capable of distinguishing between psychrotrophic *Bacillus* and mesophilic *Bacillus*. The development of an assay capable of rapidly detecting and quantifying psychrotrophic *Bacillus* in milk and other dairy products would prove invaluable to the dairy industry globally. The value to the milk industry alone, worth £3.5 billion in the UK, would be very significant indeed. The present invention addresses these needs.

It has now been discovered that the DNA encoding the major cold shock protein homologue known as cspA in *Bacillus* shows conserved nucleotide differences, at particular positions in the sequence, between psychrotrophic and mesophilic strains.

The term "conserved nucleotide base difference" as used herein means a nucleotide base difference that occurs sufficiently consistently between psychrotrophic and mesophilic strains to provide the basis for a useful test for psychrotrophic *Bacillus*. A test for psychrotrophic *Bacillus* need not necessarily be 100% accurate to be useful, although clearly 100% accuracy would be preferable. For example, a base difference which occurs 90% or 95% of the time between psychotrophs and mesophils may provide the basis for a practical test. Preferably, any inconsistencies which occur are such that false positives rather than false negatives result, so that the presence of any psychotrophs in the sample being tested does not go undetected.

The invention therefore provides in one aspect a method of testing for a psychrotrophic *Bacillus* bacterium in a sample, which method comprises determining whether the sample contains a nucleic acid which codes for a *Bacillus* major cold shock protein or major cold shock protein

homologue and which has at least one conserved nucleotide base which is different in psychrotrophic and mesophilic strains of *Bacillus*.

In a particular embodiment of the method according to the invention, the conserved nucleotide base is adenine (A) at position 4 or thymine (T) at position 9 in the nucleic acid sequence shown in Figure 1, which encodes the *Bacillus cereus* major cold shock protein homologue cspA, or both A at position 4 and T at position 9.

In another embodiment of the method according to the invention, the conserved nucleotide base is T at position 124 in the nucleic acid sequence shown in Figure 1, which encodes the *Bacillus cereus* major cold shock protein homologue cspA. It will be evident from Figure 1 that one of the sequenced mesophilic strains (designated 27877M) contains T at position 124 of the nucleotide sequence and would thus show up as a false positive in a test for psychrotrophic strains based on position 124. However, since strain 27877M represents only 1 out of the 21 mesophilic strains sequenced, position 124 is expected to provide a suitable basis for a test for psychrotrophic *Bacillus*.

In another aspect, the invention provides an oligonucleotide primer or probe, comprising at least 15 nucleotides, which is complementary to a region of the major cold shock protein DNA sequence for psychrotrophic *Bacillus cereus* shown in Figure 1, or to a region of its reverse complement, which region includes position 124 or position 4 or position 9, or both position 4 and position 9, in Figure 1.

The invention is thus concerned with methods for specifically detecting psychrotrophic *Bacillus*, and with oligonucleotides useful in such methods.

The invention is concerned primarily with detection of psychrotrophic organisms which are involved in food spoilage. The most commercially important of these is *Bacillus cereus*, but other *Bacillus* species such as *B. mycoides, B. circulans, B. coagulans, B. brevis and*

B. licheniformis are also capable of proliferating at low temperatures and are relevant to some degree to food spoilage. Major cold shock protein homologues have been found to be highly conserved throughout a range of different bacteria in which they have been sought.

In the attached drawings;

Figure 1 shows the coding region of the major cold stock protein homologue cspA gene as sequenced from various strains of psychrotrophic and mesophilic *Bacillus cereus* and from a strain of psychrotrophic *Bacillus mycoides.* Part of the 3' end of the coding region, representing about 8 amino acids, is missing.

Figure 2 shows part of the non-coding region of the cspA gene upstream of the ATG at the 5' end of the Figure 1 sequence, for the same *Bacillus cereus* strains as Figure 1.

Figure 3 shows an ethidium bromide stained agarose gel in which PCR products from a screen for psychrotrophic *Bacillus cereus* have been run (see Example 3).

The variety of different techniques which may be applied in the method according to the invention will be known to those skilled in the art. Preferably, the method according to the invention uses analysis of nucleic acid sequences. However, analysis of protein encoded by the specific nucleic acid sequences is not excluded. The substitution of A in psychrotrophic *Bacillus cereus* strains by guanine (G) in mesophilic strains at position 4 in Figure 1 is a base change which results in a different amino acid being encoded by the codon which includes the nucleotide at position 4. Mesophiles have the second codon GCA which codes for alanine, whereas psychotrophs have the second codon ACA which codes for threonine. The A-G change is therefore a significant difference, particularly since the other base differences between psychrotrophic and mesophilic strains shown in Figure 1 do not represent amino acid alterations in the protein. Analysis of the protein sequence could be carried out by lysing the

bacteria, isolating the cspA protein e.g. by means of a specific antibody directed against it, and N-terminal sequencing the protein to detect the amino acid sequence difference. Thus, differentiation between psychrotrophic and mesophilic *Bacillus* strains will also be possible at the amino acid level.

Generally, the method according to the invention will involve a nucleic acid amplification reaction. This may be performed on DNA or mRNA, or on cDNA produced from it. Amplification may be performed for example by the polymerase chain reaction (PCR). Determining whether a particular nucleic acid sequence is present may then be carried out by analysis of the amplification products, e.g. by sequencing, by the use of single-strand conformation polymorphism (SSCP) analysis, or by the use of a labelled probe which binds to the amplification reaction products. Alternatively, determining whether the specific sequence is present may be carried out as an integral part of the amplification reaction, e.g. using a probe cleavage amplification technique such as the system marketed by Perkin Elmer under the trade mark TaqMan. This is a particularly preferred technique for use in the methods according to the invention and is described in more detail below.

A preferred amplification technique for use in the method according to the invention is reverse transcriptase polymerase chain reaction (RT-PCR). RT-PCR uses the enzyme reverse transcriptase to convert mRNA to cDNA which then acts as a template for PCR. RT-PCR is advantageous over conventional PCR performed on sample DNA for two reasons. Firstly, false positives occurring due to the presence of non-viable bacteria are reduced. This is because mRNA should only be present in viable bacteria, whereas DNA is stably present in both dead and live bacteria. Secondly, major cold shock protein mRNA is highly induced at refrigeration temperatures, but inherently unstable at 37°C. This means that a sample can initially be incubated at 37°C to destroy residual

template mRNA, followed by a short incubation at 15°C or below to allow transcription of the template mRNA from viable bacteria.

The system marketed by Perkin Elmer under the trade mark TaqMan is a PCR assay system and is preferably used in the method according to the invention in combination with RT-PCR. TaqMan™ uses the hydrolysis of a fluorogenic probe to monitor the extent of amplification. The probe consists of an oligonucleotide labelled with both a fluorescent reporter and a quencher dye. During the PCR process, this probe is cleaved by the 5' nuclease activity of taq DNA polymerase if and only if it hybridises to the segment being amplified. Cleavage of the probe generates an increase in the fluorescence intensity of the reporter dye. Thus, amplification of a specific product can be detected by simply measuring fluorescence after or during PCR.

An example of a suitable probe for specifically detecting psychrotrophic *Bacillus cereus* by a conserved nucleotide base difference at position 124 in Figure 1 is CAAATCTTTAGAAGAAGGCCAAAA [SEQ ID NO: 1]. This probe will hybridise to the reverse complement of the nucleotide sequence in Figure 1, from position 117 to 141. Under suitable conditions, this or a similar probe incorporating a conserved nucleotide base difference between psychrotrophic and mesophilic *Bacillus cereus* can be used to specifically detect psychrotrophic strains.

A particularly preferred probe for detecting psychrotrophic *Bacillus cereus* is one which hybridises in a TaqMan™ assay to a region of the nucleic acid in Figure 1, or a region of its reverse complement, which region includes positions 4 and 9. An example of such a probe is Psy 1 ATGACAGTTACAGGACAAGTAAAATGGTTTAAC [SEQ ID NO: 2].

A suitable pair of primers for use in a TaqMan™ assay with the above probe is BcF2 and Bc57R or BcF2 and Bc56R with the following sequences:

BcF2 CGAATTTGATAATGTGTGGATTC [SEQ ID NO: 3]

Bc57R TCGCCTGGAACTTCGATG [SEQ ID NO: 4]

Bc56R GCTTGAGGTCCACGGTTG [SEQ ID NO: 5]

BcF2 is a forward primer which hybridises to a region upstream of the probe, to part of the non-coding region of the cspA gene upstream of the nucleotide sequence in Figure 2. Bc57R and Bc56R are reverse primers which hybridise downstream of the probe.

In addition to forward and reverse primers and a probe specific for psychrotrophic *Bacillus cereus*, a mesophile-specific probe may also be used. A suitable mesophile-specific probe for use in a TaqMan™ assay with the above probe and primers is Meso 1 which has the nucleotide sequence:

Meso 1 ATGGCAGTAACAGGACAAGTAAAATGGT [SEQ ID NO: 6]

Psychrotroph- and mesophile-specific probes may be differently labelled and used together in a TaqMan™ assay. Each will have a different fluorescent reporter which can be separately detected in order to determine whether either or both types of *Bacillus* are present in a sample.

An alternative technique for direct screening for psychrotrophic *Bacillus* uses oligonucleotide primers incorporating the conserved nucleotide base differences found between psychrotrophic and mesophilic strains, in a conventional PCR reaction. The oligonucleotide primers are designed such that a conserved nucleotide difference is incorporated at the 3' end. At least one such primer, specific for psychrotrophic *Bacillus* sequences, can be used as one of a pair of primers in a conventional PCR reaction to detect psychrotrophs. Amplified products will only result in the presence of the target sequence. Preferably, both primers in the pair are psychrotroph-specific.

Suitable specific oligonucleotide primers incorporate the conserved nucleotide base difference at position 124, or 4, or 9 of the

coding region of the cspA gene (see Figure 1) at the 3' end. For example, BcPF - 5' GGAAATAATTATGACAGTT 3' [SEQ ID NO:7] is specific for psychrotrophs and incorporates the conserved nucleotide base differences at both positions 4 and 9, position 9 being at the 3' end of the primer. BcPR - 5' CTTTTTGGCCTTCTTCTAA 3' [SEQ ID NO:8] incorporates position 124 of the sequence shown in Figure 1. The specific primers will be of a suitable length for the purpose of target specific amplification, for example between 10 and 30 nucleotides or between 15 and 25 nucleotides in length.

EXAMPLES

Example 1

TaqMan™ screening of psychrotrophic and mesophilic *Bacillus cereus*

PCR conditions

TaqMan™ PCR was performed in triplicate on 9 strains of psychrotrophic *B. cereus* (2478P, 2480P, 2482P, 2484P, 2485P, 2486P, 2487P, and 10201P) and 9 strains of mesophilic *B. cereus* (109M, 116M, 118M. 120M, 121M, 122M, 125M, 127M, and 128M), using the major cold-shock protein gene sequence *cspA* as a target. PCR was performed with a Perkin Elmer ABI 2400 automated thermocycler with 0.2 ml thin walled PCR tubes (Perkin Elmer). Reactions were carried out in 50 µl volumes containing 5µl of 10X PCR buffer (supplied with *Taq* DNA polymerase; Perkin Elmer), 4mM $MgCl_2$ (Perkin Elmer), 200 pmol of the oligonucleotide primers BcF2 (CGA ATT TGA TAA TGT GTG GAT TC [SEQ ID NO: 3]) and Bc57R (TCG CCT GGA ACT TCG ATG [SEQ ID NO: 4]), 100 pmol of the fluorogenic probes Meso 1 (TET- ATG GCA GTA ACA GGA CAA GTA AAA TGG T - TAMRA [SEQ ID NO: 6]) and Psy 1 (FAM - ATG ACA GTT ACA GGA CAA GTA AAA TGG TTT AAC - TAMRA [SEQ ID NO: 2]), 0.2 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP; Pharmacia),

1.25 U of *Taq* DNA polymerase (Perkin Elmer), and a pin head sized aliquot of bacteria picked from an agar plate of selective media. Bacterial cells were lysed by heating the mixture for 10 minutes at 95°C. Amplification of csp*A* DNA and probe cleavage was then attempted with 40 cycles at 95°C for 15 s and 62°C for 1 minute. Fluorescence was recorded from each PCR sample using a Perkin Elmer LS-50B (allelic discrimination mode).

Results

Once the LS-50B had been calibrated using a no template control, samples were read as either Homo1 (psychrotrophic strain) or Homo2 (mesophilic strain). All 18 strains of *B. cereus* were called correctly.

Example 2

Isolation of *Bacillus* from milk

It has been demonstrated that subjecting milk to a "heat - shock" of 75°C for 20 min causes the vast majority of contaminating *Bacillus* spores to germinate. Hence the following procedure can be adopted to allow the isolation of *Bacillus* from milk:

1) Collect a sample of 100-200 ml of raw milk from the milk tanker, individual farm bulk tank, dairy holding tank etc. prior to pasteurisation.

2) Heat-shock the above sample(s) at 75°C for 20 min and then rapidly cool to 30°C - 35°C.

3) Incubate milk sample(s) at 30°C - 35°C for a period of 16 - 24 hours (time period and temperature should reflect conditions necessary to give between 10^6 and 10^8 bacteria per ml of milk).

4) Place 100 µl sample(s) of the above into a 1.5 ml microcentrifuge tube containing 900 µl of warm/hot (50°C - 60°C) washing diluent (e.g. sterile water containing a mild detergent such as Triton X-100).

5) Briefly vortex the solution and pellet bacterial cells at 13,000g for 10 min.

6) Repeat washing of bacterial cells with 1 ml of warm/hot washing diluent and re-pellet bacteria as in step 5.

7) Re-suspend bacterial pellet in 100 µl of sterile water and boil for 10 min to lyse bacteria.

Quantification of Psychrotrophic *Bacillus (B. cereus/mycoides)*

1) Pipette 10 µl of cell lysate from above (step 7) into a thin walled PCR tube containing 90 µl of *B. cereus* master mix formulated as follows: 10 µl of 10X PCR buffer (supplied with *Taq* DNA polymerase), 4 mM $MgCl_2$, 200 pmol of the oligonucleotide primers BcF2 (CGA ATT TGA TAA TGT GTG GAT TC [SEQ ID NO:3]) and Bc57R (TCG CCT GGA ACT TCG ATG [SEQ ID NO: 4]), 100 pmol of the fluorogenic probe Psy 1 (FAM - ATG ACA GTT ACA GGA CAA GTA AAA TGG TTT AAC [SEQ ID NO:2]), 0.2 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP), and 1 U of *Taq* DNA polymerase.

2) Place PCR tube(s) in a thermal cycler and perform 40 cycles of 95°C for 15 s and 62°C for 1 min.

3) Use a real-time detector (e.g. ABI 7700) to monitor fluorescence and quantify starting template DNA.

4) Use above data to estimate the number of bacteria in the original sample and to estimate the quality/shelf life of the milk.

Example 3

Direct screening for psychrotrophic *B.cereus* using conventional PCR

Oligonucleotide primers with psychrotrophic *B. cereus cspA* specific sequences (BcPF - 5' GGAAATAATTATGACAGTT 3' [SEQ ID

NO: 7] and BcPR - 5' CTTTTTGGCCTTCTTCTAA 3' [SEQ ID NO: 8] were designed by incorporating the conserved nucleotide differences found between mesophilic and psychrotrophic strains of these bacteria (Figure 1). The latter primers were then used to perform PCR on genomic DNA from 10 mesophilic (109M, 116M, 118M, 120M, 121M, 122M, 125M, 127M, and 128M) and 10 psychrotrophic strains of *B. cereus* (2478P, 2480P, 2481P, 2482P, 2484P, 2485P, 2486P, 2487P, and 10201P). PCR was performed using 35 cycles of 95°C for 15 sec, 55°C for 30 sec, 72°C for 30 sec. The resulting PCR products were run on an ethidium bromide stained agarose gel (Figure 3). Only the group of 10 psychrotrophic strains gave amplified products, demonstrating the specificity of this assay for discriminating between mesophilic and psychrotrophic strains of *B. cereus*.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Biotec Laboratories Limited
(B) STREET: 32 Anson Road, Martlesham Heath
(C) CITY: Ipswich
(D) STATE: Suffolk
(E) COUNTRY: United Kingdom
(F) POSTAL CODE (ZIP): IP5 7RG

(ii) TITLE OF INVENTION: Detection of Psychrotrophic *Bacillus*

(iii) NUMBER OF SEQUENCES: 16

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: GB 9615516.3
(B) FILING DATE: 24-JUL-1996

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "synthetic DNA probe"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CAAATCTTTA GAAGAAGGCC AAAA 24

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "synthetic DNA probe"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

ATGACAGTTA CAGGACAAGT AAAATGGTTT AAC 33

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "synthetic DNA primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CGAATTTGAT AATGTGTGGA TTC 23

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "synthetic DNA primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TCGCCTGGAA CTTCGATG 18

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "synthetic DNA primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "synthetic DNA probe"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

ATGGCAGTAA CAGGACAAGT AAAATGGT 28

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "synthetic DNA primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GGAAATAATT ATGACAGTT 19

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "synthetic DNA primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CTTTTTGGCC TTCTTCTAA 19

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 171 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..171

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

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(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

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        35                  40                  45

Ser Phe Glu Ile Glu Asp Gly Asn Arg
    50                  55
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(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 171 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..171

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

```
ATG GCA GTA ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC     48
Met Ala Val Thr Gly Gln Val Lys Trp Phe Asn Asn Glu Lys Gly Phe
        60                  65                  70

GGT TTC ATC GAA GTT CCA GGC GAA AAC GAY GTA TTC GTA CAY TTC TCT     96
Gly Phe Ile Glu Val Pro Gly Glu Asn Xaa Val Phe Val Xaa Phe Ser
     75                  80                  85

GCA ATC GAA ACT GAM GGT TTC AAA TCT CTA GAA GAA GGC CAA AAA GTT    144
Ala Ile Glu Thr Xaa Gly Phe Lys Ser Leu Glu Glu Gly Gln Lys Val
 90                  95                 100                 105

AGC TTC GAA ATC GAA GAH GGY AAC CGT                             171
Ser Phe Glu Ile Glu Xaa Xaa Asn Arg
                110
```

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

```
Met Ala Val Thr Gly Gln Val Lys Trp Phe Asn Asn Glu Lys Gly Phe
  1               5                  10                  15

Gly Phe Ile Glu Val Pro Gly Glu Asn Xaa Val Phe Val Xaa Phe Ser
             20                  25                  30

Ala Ile Glu Thr Xaa Gly Phe Lys Ser Leu Glu Glu Gly Gln Lys Val
         35                  40                  45

Ser Phe Glu Ile Glu Xaa Xaa Asn Arg
     50                  55
```

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

TTTTTTWTAC ATGAAGACTA AATAAAATGT ATTTTCGTAG GAGGAAATAA TT 52

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

TTTTTTTTAC ATGAAGACTA AATAATAAAA TGTATTTTCT TAGGAGGAAA TAATT 55

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 58 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

TTTTTTAGAC AGGAAGACTA AAAAAATTAA ATTTGTATTT TCTTAGGAGG AAATAATC 58

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 59 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

TTTTTTAGAY AGGARRACTA AAAAHTTWAA AAWWTGTATT TTMTTAGGAG GAAATAATY 59

CLAIMS

1. A method of testing for a psychrotrophic *Bacillus* bacterium in a sample, which method comprises determining whether the sample contains a nucleic acid which codes for a *Bacillus* major cold shock protein or major cold shock protein homologue and which has a conserved nucleotide base which is different in psychrotrophic and mesophilic strains of *Bacillus*.

2. A method as claimed in claim 1, for testing for psychrotrophic *Bacillus* in a food sample.

3. A method as claimed in claim 1 or claim 2, which method comprises performing a nucleic acid amplification reaction on a target region of the nucleic acid which codes for a *Bacillus* major cold shock protein or major cold shock protein homologue, which target region comprises the conserved nucleotide base difference.

4. A method as claimed in any one of claims 1 to 3, which method comprises using an oligonucleotide probe which is capable under certain conditions of hybridising specifically to a nucleic acid coding for a psychrotrophic *Bacillus* major cold shock protein or major cold shock protein homologue.

5. A method as claimed in claim 4, wherein the probe is a fluorogenic probe suitable for use in a probe cleavage amplification technique.

6. A method as claimed in any one of claims 1 to 5, wherein the conserved nucleotide base is A at position 4 or T at position 9, or both A at position 4 and T at position 9, in the nucleic acid sequence in Figure 1.

7. A method as claimed in any one of claims 1 to 5, wherein the conserved nucleotide base is T at position 124 in the nucleic acid sequence in Figure 1.

8. A method as claimed in claim 6, using a probe which is complementary to a region of the major cold shock protein nucleic acid sequence for psychrotrophic *Bacillus* shown in Figure 1, or to its reverse complement, which region includes position 4 or position 9 in Figure 1, or both position 4 and position 9.

9. A method as claimed in claim 7, using a probe which is complementary to a region of the major cold shock protein nucleic acid sequence for psychrotrophic *Bacillus* shown in Figure 1, or to its reverse complement, which region includes position 124 in Figure 1.

10. An oligonucleotide probe suitable for use in a method according to any one of claims 4 to 9, comprising at least 15 nucleotides.

11. A method as claimed in claim 3, which method comprises amplifying the target region using a pair of oligonucleotide primers wherein one primer in the pair incorporates the conserved nucleotide base difference at its 3' end.

12. A method as claimed in claim 11, wherein a second primer in the pair incorporates a second conserved nucleotide base difference at its 3' end.

13. A method as claimed in claim 11 or claim 12, wherein the pair of oligonucleotide primers is capable of specifically amplifying psychrotrophic *Bacillus* nucleic acid.

14. A method as claimed in any one of claims 11 to 13, wherein the conserved nucleotide base difference is at position 4 or position 9 or position 124 in the nucleic acid sequence in Figure 1.

15. A pair of oligonucleotide primers suitable for use in a method according to any one of claims 11 to 14.

Figure 1

5' coding region

```
        M   T   V   T   G   Q   V   K   W   F   N   N   E   K   G   F   G   F   I

10201P  ATG ACA GTT ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC GGT TTC ATC
2477P   ATG ACA GTT ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC GGT TTC ATC
2478P   ATG ACA GTT ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC GGT TTC ATC
2480P   ATG ACA GTT ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC GGT TTC ATC
2481P   ATG ACA GTT ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC GGT TTC ATC
2482P   ATG ACA GTT ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC GGT TTC ATC
2484P   ATG ACA GTT ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC GGT TTC ATC
2485P   ATG ACA GTT ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC GGT TTC ATC
2486P   ATG ACA GTT ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC GGT TTC ATC
2487P   ATG ACA GTT ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC GGT TTC ATC
B.my    ATG ACA GTT ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC GGT TTC ATC


        M   A   V   T   G   Q   V   K   W   F   N   N   E   K   G   F   G   F   I

109M    ATG GCA GTA ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC GGT TTC ATC
116M    ATG GCA GTA ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC GGT TTC ATC
118M    ATG GCA GTA ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC GGT TTC ATC
120M    ATG GCA GTA ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC GGT TTC ATC
121M    ATG GCA GTA ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC GGT TTC ATC
122M    ATG GCA GTA ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC GGT TTC ATC
125M    ATG GCA GTA ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC GGT TTC ATC
126M    ATG GCA GTA ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC GGT TTC ATC
127M    ATG GCA GTA ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC GGT TTC ATC
128M    ATG GCA GTA ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC GGT TTC ATC
129M    ATG GCA GTA ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC GGT TTC ATC
130M    ATG GCA GTA ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC GGT TTC ATC
131M    ATG GCA GTA ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC GGT TTC ATC
134M    ATG GCA GTA ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC GGT TTC ATC
135M    ATG GCA GTA ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC GGT TTC ATC
144M    ATG GCA GTA ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC GGT TTC ATC
1399M   ATG GCA GTA ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC GGT TTC ATC
1414M   ATG GCA GTA ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC GGT TTC ATC
7064M   ATG GCA GTA ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC GGT TTC ATC
27877M  ATG GCA GTA ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC GGT TTC ATC
211B    ATG GCA GTA ACA GGA CAA GTA AAA TGG TTT AAC AAC GAA AAA GGC TTC GGT TTC ATC
```

Figure 1 (cont)

	E	V	P	G	E	N	D	V	F	V	H	F	S	A	I	E	T	D/E	G
10201P	GAA	GTT	CCA	GGC	GAA	AAC	GAT	GTA	TTC	GTA	CAT	TTC	TCT	GCA	ATC	GAA	ACT	GAT	GGT
2477P	GAA	GTT	CCA	GGC	GAA	AAC	GAT	GTA	TTC	GTA	CAT	TTC	TCT	GCA	ATC	GAA	ACT	GAT	GGT
2478P	GAA	GTT	CCA	GGC	GAA	AAC	GAT	GTA	TTC	GTA	CAT	TTC	TCT	GCA	ATC	GAA	ACT	GAT	GGT
2480P	GAA	GTT	CCA	GGC	GAA	AAC	GAT	GTA	TTC	GTA	CAT	TTC	TCT	GCA	ATC	GAA	ACT	GAT	GGT
2481P	GAA	GTT	CCA	GGC	GAA	AAC	GAT	GTA	TTC	GTA	CAT	TTC	TCT	GCA	ATC	GAA	ACT	GAT	GGT
2482P	GAA	GTT	CCA	GGC	GAA	AAC	GAT	GTA	TTC	GTA	CAT	TTC	TCT	GCA	ATC	GAA	ACT	GAT	GGT
2484P	GAA	GTT	CCA	GGC	GAA	AAC	GAT	GTA	TTC	GTA	CAT	TTC	TCT	GCA	ATC	GAA	ACT	GAA	GGT
2485P	GAA	GTT	CCA	GGC	GAA	AAC	GAT	GTA	TTC	GTA	CAT	TTC	TCT	GCA	ATC	GAA	ACT	GAC	GGT
2486P	GAA	GTT	CCA	GGC	GAA	AAC	GAT	GTA	TTC	GTA	CAT	TTC	TCT	GCA	ATC	GAA	ACT	GAT	GGT
2487P	GAA	GTT	CCA	GGC	GAA	AAC	GAT	GTA	TTC	GTA	CAT	TTC	TCT	GCA	ATC	GAA	ACT	GAT	GGT
B.my	GAA	GTT	CCA	GGC	GAA	AAC	GAT	GTA	TTC	GTA	CAT	TTC	TCT	GCA	ATC	GAA	ACT	GAT	GGT

	E	V	P	G	E	N	D	V	F	V	H	F	S	A	I	E	T	D/E	G
109M	GAA	GTT	CCA	GGC	GAA	AAC	GAC	GTA	TTC	GTA	CAC	TTC	TCT	GCA	ATC	GAA	ACT	GAA	GGT
116M	GAA	GTT	CCA	GGC	GAA	AAC	GAC	GTA	TTC	GTA	CAC	TTC	TCT	GCA	ATC	GAA	ACT	GAA	GGT
118M	GAA	GTT	CCA	GGC	GAA	AAC	GAC	GTA	TTC	GTA	CAC	TTC	TCT	CGA	ATC	GAA	ACT	GAA	GGT
120M	GAA	GTT	CCA	GGC	GAA	AAC	GAT	GTA	TTC	GTA	CAT	TTC	TCT	GCA	ATC	GAA	ACT	GAC	GGT
121M	GAA	GTT	CCA	GGC	GAA	AAC	GAT	GTA	TTC	GTA	CAT	TTC	TCT	GCA	ATC	GAA	ACT	GAC	GGT
122M	GAA	GTT	CCA	GGC	GAA	AAC	GAC	GTA	TTC	GTA	CAC	TTC	TCT	GCA	ATC	GAA	ACT	GAA	GGT
125M	GAA	GTT	CCA	GGC	GAA	AAC	GAC	GTA	TTC	GTA	CAC	TTC	TCT	GCA	ATC	GAA	ACT	GAA	GGT
126M	GAA	GTT	CCA	GGC	GAA	AAC	GAC	GTA	TTC	GTA	CAC	TTC	TCT	GCA	ATC	GAA	ACT	GAA	GGT
127M	GAA	GTT	CCA	GGC	GAA	AAC	GAC	GTA	TTC	GTA	CAC	TTC	TCT	GCA	ATC	GAA	ACT	GAA	GGT
128M	GAA	GTT	CCA	GGC	GAA	AAC	GAC	GTA	TTC	GTA	CAT	TTC	TCT	GCA	ATC	GAA	ACT	GAC	GGT
129M	GAA	GTT	CCA	GGC	GAA	AAC	GAC	GTA	TTC	GTA	CAT	TTC	TCT	GCA	ATC	GAA	ACT	GAC	GGT
130M	GAA	GTT	CCA	GGC	GAA	AAC	GAC	GTA	TTC	GTA	CAT	TTC	TCT	GCA	ATC	GAA	ACT	GAC	GGT
131M	GAA	GTT	CCA	GGC	GAA	AAC	GAC	GTA	TTC	GTA	CAT	TTC	TCT	GCA	ATC	GAA	ACT	GAC	GGT
134M	GAA	GTT	CCA	GGC	GAA	AAC	GAT	GTA	TTC	GTA	CAT	TTC	TCT	GCA	ATC	GAA	ACT	GAC	GGT
135M	GAA	GTT	CCA	GGC	GAA	AAC	GAT	GTA	TTC	GTA	CAT	TTC	TCT	GCA	ATC	GAA	ACT	GAC	GGT
144M	GAA	GTT	CCA	GGC	GAA	AAC	GAT	GTA	TTC	GTA	CAT	TTC	TCT	GCA	ATC	GAA	ACT	GAC	GGT
1399M	GAA	GTT	CCA	GGC	GAA	AAC	GAT	GTA	TTC	GTA	CAC	TTC	TCT	GCA	ATC	GAA	ACT	GAA	GGT
1414M	GAA	GTT	CCA	GGC	GAA	AAC	GAC	GTA	TTC	GTA	CAT	TTC	TCT	GCA	ATC	GAA	ACT	GAC	GGT
7064M	GAA	GTT	CCA	GGC	GAA	AAC	GAC	GTA	TTC	GTA	CAT	TTC	TCT	GCA	ATC	GAA	ACT	GAC	GGT
27877M	GAA	GTT	CCA	GGC	GAA	AAC	GAC	GTA	TTC	GTA	CAC	TTC	TCA	GCA	ATC	GAA	ACT	GAC	GGT
211B	GAA	GTT	CCA	GGC	GAA	AAC	GAC	GTA	TTC	GTA	CAC	TTC	TCT	GCA	ATC	GAA	ACT	GAA	GGT

Figure 1 (cont)

```
        F   K   S   L   E   E   G   Q   K   V   S   F   E   I   E   D/E G   N   R
                                :                   .                       .   .
10201P  TTC AAA TCT TTA GAA GAA GGC CAA AAA GTT AGC TTC GAA ATC GAA GAT GGC AAC CGT
2477P   TTC AAA TCT TTA GAA GAA GGC CAA AAA GTT AGC TTC GAA ATC GAA GAT GGC AAC CGT
2478P   TTC AAA TCT TTA GAA GAA GGC CAA AAA GTT AGC TTC GAA ATC GAA GAT GGC AAC CGT
2480P   TTC AAA TCT TTA GAA GAA GGC CAA AAA GTT AGC TTC GAA ATC GAA GAT GGC AAC CGT
2481P   TTC AAA TCT TTA GAA GAA GGC CAA AAA GTT AGC TTC GAA ATC GAA GAT GGC AAC CGT
2482P   TTC AAA TCT TTA GAA GAA GGC CAA AAA GTT AGC TTC GAA ATC GAA GAT GGC AAC CGT
2484P   TTC AAA TCT TTA GAA GAA GGC CAA AAA GTT AGC TTC GAA ATC GAA GAA GGC AAC CGT
2485P   TTC AAA TCT TTA GAA GAA GGC CAA AAA GTT AGC TTC GAA ATC GAA GAT GGC AAC CGT
2486P   TTC AAA TCT TTA GAA GAA GGC CAA AAA GTT AGC TTC GAA ATC GAA GAT GGC AAC CGT
2487P   TTC AAA TCT TTA GAA GAA GGC CAA AAA GTT AGC TTC GAA ATC GAA GAT GGC AAC CGT
B.my    TTC AAA TCT TTA GAA GAA GGC CAA AAA GTT AGC TTC GAA ATC GAA GAT GGC AAC CGT
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PSYCHROTROPHS

```
        F   K   S   L   E   E   G   Q   K   V   S   F   E   I   E   D/E G   N   R
109M    TTC AAA TCT CTA GAA GAA GGC CAA AAA GTT AGC TTC GAA ATC GAA GAA GGT AAC CGT
116M    TTC AAA TCT CTA GAA GAA GGC CAA AAA GTT AGC TTC GAA ATC GAA GAA GGT AAC CGT
118M    TTC AAA TCT CTA GAA GAA GGT CAA AAA GTT AGC TTC GAA ATC GAA GAA GGT AAC CGT
120M    TTC AAA TCT CTA GAA GAA GGT CAA AAA GTT AGC TTC GAA ATC GAA GAT GGC AAC CGT
121M    TTC AAA TCT CTA GAA GAA GGT CAA AAA GTT AGC TTC GAA ATC GAA GAT GGC AAC CGT
122M    TTC AAA TCT CTA GAA GAA GGT CAA AAA GTT AGC TTC GAA ATC GAA GAA GGT AAC CGT
125M    TTC AAA TCT CTA GAA GAA GGT CAA AAA GTT AGC TTC GAA ATC GAA GAA GGT AAC CGT
126M    TTC AAA TCT CTA GAA GAA GGC CAA AAA GTT AGC TTC GAA ATC GAA GAA GGT AAC CGT
127M    TTC AAA TCT CTA GAA GAA GGC CAA AAA GTT AGC TTC GAA ATC GAA GAA GGT AAC CGT
128M    TTC AAA TCT CTA GAA GAA GGT CAA AAA GTT AGC TTC GAA ATC GAA GAT GGT AAC CGT
129M    TTC AAA TCT CTA GAA GAA GGT CAA AAA GTT AGC TTC GAA ATC GAA GAT GGT AAC CGT
130M    TTC AAA TCT CTA GAA GAA GGT CAA AAA GTT AGC TTC GAA ATC GAA GAT GGT AAC CGT
131M    TTC AAA TCT CTA GAA GAA GGT CAA AAA GTT AGC TTC GAA ATC GAA GAT GGT AAC CGT
134M    TTC AAA TCT CTA GAA GAA GGT CAA AAA GTT AGC TTC GAA ATC GAA GAT GGC AAC CGT
135M    TTC AAA TCT CTA GAA GAA GGT CAA AAA GTT AGC TTC GAA ATC GAA GAT GGC AAC CGT
144M    TTC AAA TCT CTA GAA GAA GGT CAA AAA GTT AGC TTC GAA ATC GAA GAT GGC AAC CGT
1399M   TTC AAA TCT CTA GAA GAA GGC CAA AAA GTT AGC TTC GAA ATC GAA GAA GGT AAC CGT
1414M   TTC AAA TCT CTA GAA GAA GGT CAA AAA GTT AGC TTC GAA ATC GAA GAT GGT AAC CGT
7064M   TTC AAA TCT CTA GAA GAA GGT CAA AAA GTT AGC TTC GAA ATC GAA GAA GGT AAC CGT
27877M  TTC AAA TCT TTA GAA GAA GGT CAA AAA GTT AGC TTC GAA ATC GAA GAC GGT AAC CGT
211B    TTC AAA TCT CTA GAA GAA GGC CAA AAA GTT AGC TTC GAA ATC GAA GAA GGT AAC CGT
```

MESOPHILES

Nucleotide and amino acid sequences for psychrotrophs in SEQ ID NO: 9 and 10

Nucleotide and amino acid sequences for mesophiles in SEQ ID No: 11 and 12

Figure 2

5' non-coding region

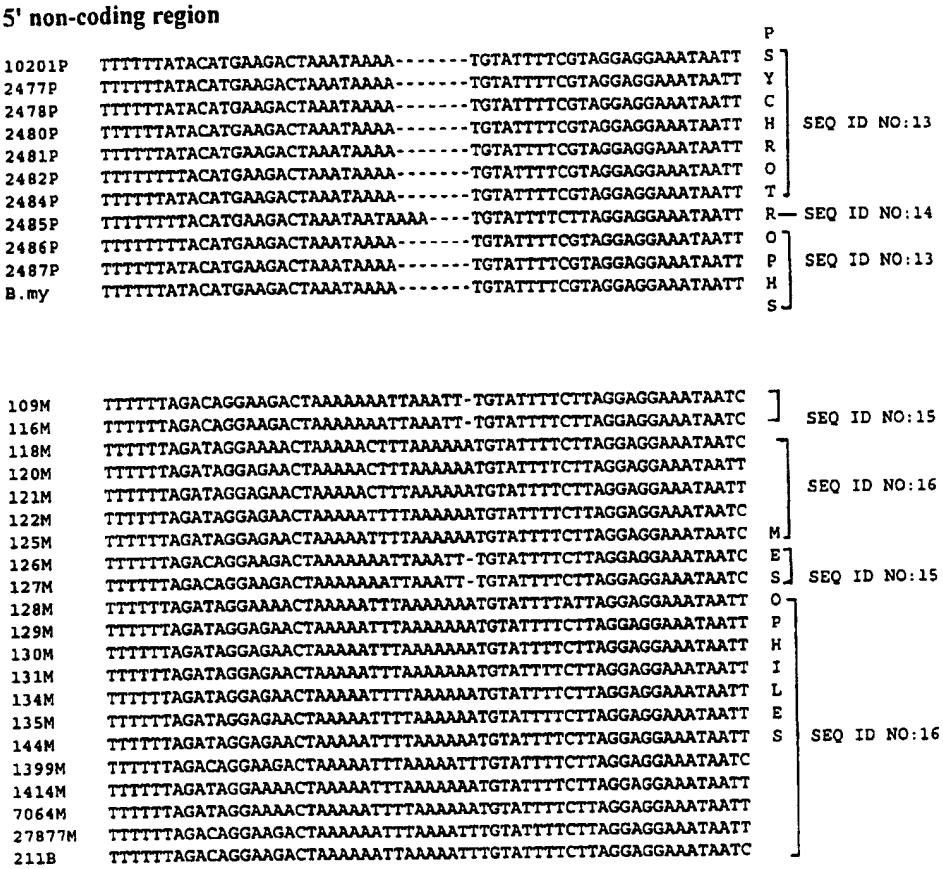
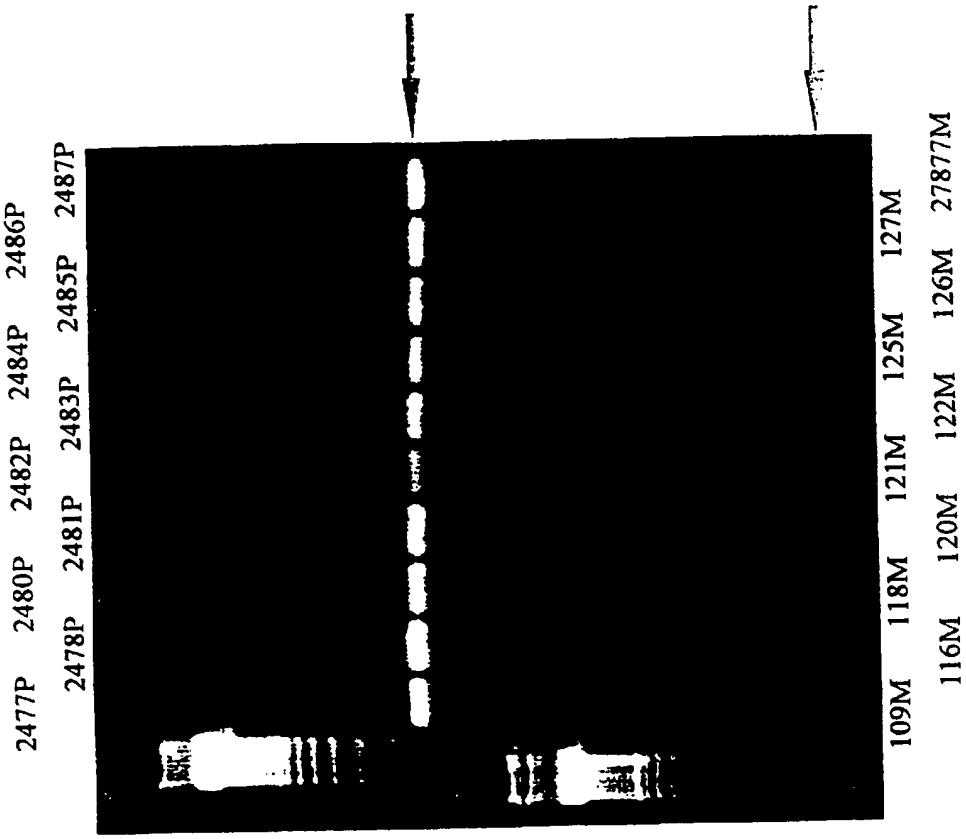


Figure 3


2477P
2480P
2478P
2482P
2481P
2484P
2483P
2486P
2485P
2487P
109M
116M
118M
120M
121M
122M
125M
126M
127M
27877M

INTERNATIONAL SEARCH REPORT

Interna ial Application No
PCT/GB 97/01987

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C07K14/32

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MAYR ET AL.: "Identification and purification of a family of dimeric major cold shock protein homologs from the psychotropic bacillus cereus WSBC 10201" JOURNAL OF BACTERIOLOGY, vol. 178, no. 10, May 1996, pages 2916-2925, XP002047488 see the whole document ---	1-15
A	SCHRÖDER ET AL.: "Mapping of the Bacillus subtilis cspB gene and cloning of its homologs in thermophilic, mesophilic and psychotropic bacilli" GENE, vol. 136, no. 1-2, 1993, pages 277-280, XP002047489 --- -/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
19 November 1997	0 9 -12- 1997

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Ceder, O

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INTERNATIONAL SEARCH REPORT

Intern. ial Application No
PCT/GB 97/01987

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 395 292 A (BARRY THOMAS GERARD ;GANNON BERNARD FRANCIS XAVIER (IE); IRELAND B) 31 October 1990 ---	
A	WO 96 15264 A (JSD TECH LTD ;STEWART GORDON SYDNEY ANDERSON (GB); FRANCIS KEVIN P) 23 May 1996 -----	

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interna al Application No
PCT/GB 97/01987

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0395292 A	31-10-90	AT 150091 T	15-03-97
		AU 630932 B	12-11-92
		AU 5365290 A	25-10-90
		DE 69030131 D	17-04-97
		DE 69030131 T	17-07-97
		ES 2100161 T	16-06-97
		JP 3130099 A	03-06-91
		US 5574145 A	12-11-96
WO 9615264 A	23-05-96	EP 0731850 A	18-09-96